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Single-Molecule Studies of the Replisome

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Single-Molecule Studies of the Replisome

Antoine M. van Oijen and Joseph J. Loparo

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115; email: antoine.van_oijen@hms.harvard.edu

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Abstract

Replication of DNA is carried out by the replisome, a multiprotein complex responsible for the unwinding of parental DNA and the synthesis of DNA on each of the two DNA strands. The impressive speed and processivity with which the replisome duplicates DNA are a result of a set of tightly regulated interactions between the replication proteins. The transient nature of these protein interactions makes it challenging to study the dynamics of the replisome by ensemble-averaging techniques. This review describes single-molecule methods that allow the study of individual replication proteins and their functioning within the replisome. The ability to mechanically manipulate individual DNA molecules and record the dynamic behavior of the replisome while it duplicates DNA has led to an improved understanding of the molecular mechanisms underlying DNA replication.

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INTRODUCTION

The replisome is the molecular machinery responsible for the replication of parental DNA into two identical daughter molecules. In all three domains of life, replisomes operate according to a set of highly conserved principles (**Figure 1**) (4, 5, 40, 80). First, parental double-stranded DNA (dsDNA) is unwound by a helicase into two DNA strands (87, 88). Two DNA polymerases, complexed with processivity factors, subsequently synthesize DNA on each of the two single-stranded templates (11, 51, 89). Because DNA polymerases add nucleotides to a primer only in the 5' to 3' direction and the two single-stranded DNA (ssDNA) templates are of opposite polarity, one of the DNA polymerases, the leading-strand polymerase, synthesizes DNA in a continuous fashion while following the helicase. The second polymerase, the lagging-strand polymerase, synthesizes short stretches of DNA in the opposite direction. The discontinuous synthesis of DNA on the lagging strand is primed by short RNA primers produced by a DNA primase (30) and gives rise to a succession of

Okazaki fragments that are later ligated into one continuous strand. Single-stranded DNA binding proteins (SSBs) remove any secondary structure that may inhibit synthesis and protect the stretches of transiently exposed ssDNA from nucleolytic attacks (96). SSBs also play a key role in mediating interactions between the various components of the replisome (40, 96, 109).

The speed and accuracy of the replisome illustrate the efficient coordination of its components. For *Escherichia coli*, the replication fork moves at a rate approaching 1000 nucleotides per second (16) and makes less than one mistake per 10^9 nucleotide incorporations (26). Once every few seconds, the primase synthesizes a short primer that is rapidly transferred to the DNA polymerase and extended into an Okazaki fragment (124). Various specific interactions between the proteins at the replication fork give the replisome its efficiency by greatly increasing the activity of the individual components. In particular, the lagging-strand DNA polymerase remains associated with the replisome, allowing it to be recycled for every new Okazaki fragment (15, 22, 54, 99). This arrangement is facilitated by a looping-back of the lagging strand to the replication machinery (2). The loop grows and collapses with each cycle of Okazaki fragment synthesis.

The various enzymatic events involved in Okazaki fragment synthesis must occur with such organization that lagging-strand synthesis remains in step with the continuous production of DNA on the leading strand. This organization requires a precisely timed series of enzymatic events that control the synthesis of a primer, the recycling of the lagging-strand DNA polymerase, and the production of an Okazaki fragment. To understand the mechanisms controlling the coordination of the different proteins at the replication fork, there is a need to directly probe the dynamics of fully functional replisomes during the replication reaction. The recent development of single-molecule tools has allowed such studies and has already led to remarkable contributions to our understanding of the

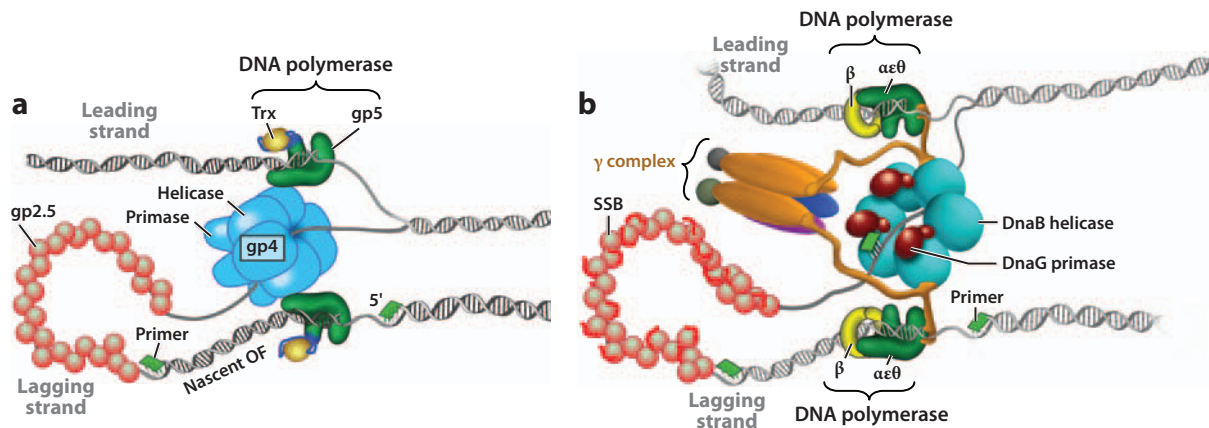


Figure 1

Schematic representations of the replisomes of the T7 bacteriophage (panel *a*) and *Escherichia coli* (panel *b*). The parental DNA is unwound by a helicase (gp4 or DnaB for the T7 or *E. coli* system, respectively), and nucleotides are synthesized on the single-stranded DNA (ssDNA) product strands by the DNA polymerases. In the T7 replisome, the DNA polymerases are directly associated with the helicase, whereas in the *E. coli* system, the DNA polymerases are tethered to the helicase by the γ clamp-loader complex. The γ complex also serves to load the β processivity clamps onto the DNA. The T4 replication machinery (not shown here) displays similarities to both the T7 and *E. coli* systems: It contains a dimeric DNA polymerase complex that is directly anchored to the helicase, and it utilizes a clamp loader to assemble processivity clamps onto the DNA. (Panels *a* and *b* reproduced with permission from References 39 and 104, respectively. OF, Okazaki fragment.

replication machinery. The ability to record molecular movies of enzymes at the single-molecule level, and thus remove averaging over large numbers of molecules participating in the reaction, has provided unique insights into their dynamics and reaction mechanisms (10, 20, 33, 50, 73, 75, 107, 120, 127).

The robustness of DNA as a substrate and the development of techniques to manipulate individual DNA molecules (reviewed in Reference 13) have led to an important role for single-molecule biophysics in understanding how nucleic acid enzymes work (27, 35, 41, 92, 94). This review aims to describe recent contributions made by single-molecule studies to our understanding of the functioning of the protein complexes involved in replication. First, single-molecule studies on the individual components of the replication machinery are discussed. Second, a description of the progress made toward obtaining a single-molecule view of the entire replisome is provided. This review concludes with a discussion of how single-molecule tools can contribute to addressing the important outstanding problems in the field and what

technical barriers need to be overcome to answer these questions.

SYNTHESIZING DNA: THE DNA POLYMERASE

DNA polymerases catalyze the synthesis of a new DNA strand and, with few exceptions, use a single-stranded nucleic acid as a template for this reaction (11, 51, 89). Both prokaryotic and eukaryotic cells contain multiple DNA polymerases, each specialized to carry out tasks in DNA replication, recombination, and repair. DNA polymerases extend a DNA chain by catalyzing the nucleophilic attack of the 3'-OH end of the growing chain on the α -phosphate of an incoming deoxyribonucleoside triphosphate (dNTP) complementary to the template. Most known replicative DNA polymerases perform this reaction in a processive manner, incorporating thousands of nucleotides without dissociating from its template. The bacterial replicative DNA polymerases possess a 3'-5' exonucleolytic activity that proceeds in the reverse direction of DNA synthesis. This activity

provides proofreading capability by removing a mismatched penultimate nucleotide, resulting in an overall incorporation accuracy of one mistake for every 10^5 – 10^7 nucleotides.

The low error rate of DNA polymerases cannot be explained by the thermodynamics of base pairing alone. Instead, several kinetic and structural studies have demonstrated that the rate, processivity, and efficiency of proofreading all strongly depend on subtle conformational variations within the enzyme's active site (25, 49, 115). Across families and even in the absence of sequence homology, DNA polymerases have a similar structure that consists of a right-hand fold, with the template DNA threaded through the palm domain and the thumb and fingers closing around it.

During incorporation of a nucleotide, the enzyme proceeds through a series of conformational changes that correspond to the closing and opening of its hand-shaped structure. Prior to the chemistry step, a DNA polymerase must bind the primer template and undergo a conformational change that requires an incoming nucleotide. Using a single-molecule fluorescence assay that exploited the sensitivity of the fluorophore Cy3 to its local environment, the Xie group (70) probed this conformational change for the bacteriophage T7 polymerase. They determined that this conformational transition involves the closing of the fingers domain and that it slows significantly for an improperly matched incoming dNTP (70). Therefore, this conformational change, which is required for catalysis, serves as a kinetic checkpoint for proper base pairing.

The ability to observe a single DNA polymerase moving along DNA while incorporating nucleotides allows for a more extensive study of the relation between enzyme template interaction and kinetics (71, 117). Single-molecule tools have been particularly powerful in elucidating the mechanisms underlying the switching between polymerization and exonuclease modes. By using optical (76) or magnetic (100) tweezers, a stretching force can be exerted on a primed single-stranded template and the influence of the force on the

polymerization rate investigated (44, 71, 117). For several prokaryotic polymerases, forces higher than 30–40 pN stall the enzyme (44, 71, 117). Single-molecule studies on the DNA polymerase of the T7 and Φ 29 bacteriophages showed that even higher forces stimulate the exonuclease activity by several orders of magnitude (44, 117). The response of the enzyme's translocation rates to a variation of the load applied provides information on which biochemical steps in the pathway are coupled to movement and how an external stretching force influences the partitioning between the exonuclease and polymerization modes (31, 32, 44, 117).

After incorporation of a correct nucleotide, the polymerase moves a distance of one base toward the site of the next incorporation. During this movement, the enzyme is briefly associated with the DNA only by electrostatic interactions with the DNA backbone and transiently has a higher probability to dissociate. To maintain a high affinity for the primer template throughout the entire catalytic cycle of synthesis and translocation, replicative DNA polymerases employ processivity factors that interact with both the DNA and the polymerase (9, 42, 46). In recent years, researchers have used single-molecule techniques to study the interaction between processivity factors and DNA by visualizing how these protein factors move along DNA in the absence of their partner polymerase (56, 57). By fluorescently labeling the processivity factor and using total internal reflection fluorescence imaging to visualize its position along a stretched duplex DNA, the nonspecific interaction of these factors with DNA gives rise to a diffusive motion along the duplex.

The kinetics of diffusive translocation under various conditions, such as salt strength and strength of fluid flow, report on the microscopic details of the interactions between the processivity factor and DNA. Application of these techniques to the eukaryotic processivity factor PCNA and that of the herpes simplex virus DNA polymerase showed that these proteins move along the DNA through a combination of sliding, while maintaining continuous

contact with the backbone, and hopping by a series of rapid and local dissociation and rebinding events (56, 57). Even though these studies are performed in the absence of DNA polymerases, the new insight they provide into how processivity factors interact with DNA is relevant to our understanding of how DNA polymerases hang onto the DNA when translocating from one incorporation site to the next on the 3' end of a primer.

UNWINDING DOUBLE-STRANDED DNA: THE HELICASE

DNA helicases are enzymes capable of unwinding duplex DNA to provide the ssDNA templates that are required in many biological processes (87, 88). Helicases separate the strands of a double helix using the energy derived from nucleotide hydrolysis. Single-molecule techniques that allow the application of a well-defined stretching force on a forked DNA substrate are well suited to monitor rates and processivities of unwinding and to study their dependency on the applied load. Well-known examples of prokaryotic replicative helicases include DnaB from *E. coli*, gp4 from the bacteriophage T7, and gp41 from T4, all of which are hexameric, donut-shaped proteins belonging to the DnaB superfamily (7).

Extensive biochemical and structural characterization led to the conclusion that these proteins encircle a single strand of DNA and translocate from the 5' to 3' direction (88). Upon encountering a ssDNA/dsDNA junction, the complementary strand is displaced and the dsDNA unwound. However, it is not well understood how the ssDNA translocation activity is coupled to the unwinding. Two different models are typically considered: active or passive coupling (6, 23, 69). A passive helicase acts as a Brownian ratchet; it waits for a thermal fluctuation that transiently melts the first few base pairs of the dsDNA, and then moves forward and binds to the newly available ssDNA. The active model describes a helicase that employs an irreversible powerstroke to disrupt the

dsDNA. In this case, the hydrolysis of nucleotides is tightly coupled to the destabilization of the duplex, leading the helicase to unwind the dsDNA without being significantly slowed down by the ssDNA/dsDNA barrier.

Johnson et al. (48) employed force to study the kinetics of T7 gp4 unwinding of dsDNA and translocation on ssDNA at the single-molecule level. By tethering the 3' terminus of one end of the DNA to a surface and the 5' terminus of the same end to an optically trapped bead, forces that assist the unwinding of the dsDNA can be applied. The rate of DNA translocation was measured by sensing the force change when the translocating helicase on ssDNA approaches a replication fork, and the rate of unwinding was measured using feedback control of force to follow the fork movement. To determine whether the helicase acts in a passive or active way, the rate of DNA unwinding was monitored at different unzipping forces. The relation between the unwinding rate and the unzipping force provided direct evidence that the gp4 helicase unwinds DNA in an active mode in which the helicase partially destabilizes the fork junction to facilitate unwinding. Studies of DNA hairpin unwinding by individual T4 gp41 proteins, the T4 phage replicative helicase, demonstrated a purely passive unwinding mechanism (68). Future experiments on other systems are needed to elucidate the significance of variation between the different helicases in the unwinding mechanism.

SINGLE-STRANDED DNA-BINDING PROTEINS

Single-stranded DNA-binding proteins (SSBs) are essential cofactors in a large number of processes involving DNA, including replication and recombination (60). An obvious, early recognized role of SSBs is the coating of the ssDNA that is transiently exposed at the lagging strand during replication to protect it from nucleolytic degradation (59). SSBs play an important regulatory role within the replisome by interacting with other replication proteins (40, 96, 109).

The thermodynamic and kinetic properties of ssDNA coating and dsDNA destabilization by SSBs are ideally suited for investigation by single-molecule tools (74). Using optical tweezers, the Williams group studied the effects of the T4 gp32 and the T7 gp2.5 ssDNA binding proteins on the melting behavior of duplex DNA (83–85, 97). They used these data to understand the mechanisms of interaction between SSBs and DNA. Thermodynamically analogous to the melting temperature of dsDNA, a critical force exists at which duplex DNA is converted into two ssDNA molecules. The influence of SSBs on this melting force provides information on the binding affinity of the protein to ssDNA. In contrast to temperature-induced melting, these force studies can be performed at physiological temperatures, thus avoiding protein denaturation. The dependency of the SSB equilibrium binding constant as a function of the ionic strength revealed that the binding of these proteins to ssDNA is regulated by electrostatically sensitive intramolecular conformational changes.

THE REPLISOME: A MULTIENZYME REPLICATION MACHINERY

In the previous sections we have described how single-molecule methods have shed light on how individual components of the replisome function. Yet, for even the simplest replication model systems these proteins do not work in isolation. Instead, dynamic protein-protein interactions between the components greatly stimulate their individual activities. Such cooperative behavior is not surprising, as the cell likely uses it as a crude method of regulation; individual replicative helicases and polymerases operating outside the context of the replisome could potentially damage the genome. Furthermore, the protein-protein interactions within the replisome act to coordinate the activity on the leading and lagging strands.

To understand how the various replisomal protein activities are coordinated, a kinetic and quantitative characterization of the many

transient intermediates involved in DNA replication is needed. Recent advances in imaging and molecular manipulation techniques have made it possible to monitor individual replication machineries and observe some of their salient dynamic features. In the remainder of this review, we discuss these studies and how they aid in obtaining a full kinetic characterization of the various enzymatic steps involved in replication.

Assembly of the Replisome

Before replication is initiated, the various components of the replication machinery are assembled at origins of replication. The assembly of multiprotein complexes does not necessarily occur in a well-defined sequential process. Instead, assembly can occur over multiple parallel pathways containing a wide range of transition rates. Determining the order in which the various proteins assemble is difficult, if not impossible, to monitor using ensemble-averaged experiments. Numerous order-of-assembly questions arise for replication systems that require accessory proteins for loading processivity clamps and helicases onto DNA (8, 21, 45, 46, 58, 81). For example, does the clamp loader associate with a clamp in solution, or does it first bind to DNA before recruiting a clamp? Is the DNA polymerase already associated with the clamp when assembled to the DNA or does a loaded clamp recruit polymerase from solution?

The T4 bacteriophage replisome represents the simplest replication model system utilizing sliding clamp–DNA polymerase complexes for which the clamp requires a clamp-loader complex. The order of assembly of labeled T4 replication proteins was visualized by measuring fluorescence resonance energy transfer (FRET) between the different proteins during their association with forked substrates (98, 118, 119, 125). This work demonstrated that the T4 DNA polymerase can be assembled through one of four major pathways (98). These different routes of assembly each may play a role in

distinct phases of the replication cycle, such as leading- and lagging-strand synthesis.

Using these single-molecule FRET imaging techniques, the authors also investigated how the hexameric T4 helicase is assembled around the DNA and, when in the assembly pathway, interacts with the hexameric T4 primase (125). DNA polymerase activity was inhibited until the helicase loading protein had completed its job of assembling the helicase (118, 119). By taking snapshots under various assembly conditions, these experiments provide invaluable information on equilibrium binding stoichiometries. An important future direction will be the real-time observation of the assembly process, allowing the determination of kinetic parameters.

Leading-Strand Synthesis

The mechanical stretching of individual DNA molecules by means of optical trapping or magnetic tweezing is particularly powerful in the study of replisomal DNA polymerase (70, 71, 117) and helicase (48, 68) activity. However, with some exceptions (91), these techniques typically do not allow for the simultaneous observation of multiple reactions. This multiplexing is essential in gathering statistically significant sample sizes and observing the low-probability events associated with the activity of multiprotein complexes.

To observe many replication reactions simultaneously, a DNA-stretching technique based on hydrodynamic flow has been developed that allows as many as hundreds of individual replication reactions to be observed in one experiment (106, 113). Individual λ phage DNA molecules are attached at one end to the bottom surface of a glass flow cell and at the opposite end to polystyrene beads. When a laminar flow is applied above the surface, a force proportional to the flow rate and the diameter of the polymer bead stretches the DNA molecules. The parabolic flow profile, with a zero flow rate at the surface, keeps the beads just above the surface. The DNA molecules are stretched by small forces of only a few piconewtons, too

small to significantly influence protein-protein and protein-DNA interactions.

As a first step toward observing the activity of a fully assembled replisome at the single-molecule level, leading-strand synthesis of the bacteriophage T7 replication system was recorded (64). The orchestration between the various components at the replication fork can be studied with the replication proteins of bacteriophage T7 (**Figure 1a**) (40). Its replisome can be reconstituted with as little as four proteins and displays all the important features of more complicated replication systems: speed, accuracy, and processivity. The T7 DNA polymerase consists of a 1:1 complex of the T7 gene 5 protein (gp5), encoded by the phage, and the thioredoxin processivity factor, encoded by the *E. coli* host (103). The T7 gene 4 protein (gp4) provides both helicase and primase activities (28, 34). The helicase activity, required for unwinding the parental DNA strand, is located in the C-terminal half, and the primase activity, required to initiate lagging-strand DNA synthesis, is located in the N-terminal half.

When both T7 DNA polymerase and gp4 are present at the fork, their dissociation from DNA is dramatically slowed (78). This results in a highly processive unwinding of the DNA duplex and synthesis on the leading strand. T7 DNA polymerase is stably bound to gp4 and converts the 3'-single-stranded product arising from the gp4 helicase activity into dsDNA by extending a short DNA primer that is annealed to the leading strand. In the absence of ribonucleotides, the primase domain on gp4 is not active and the 5' strand (the lagging strand) remains in the single-stranded form. The coiling of ssDNA causes it to be shorter than dsDNA at low stretching forces (<6 pN; see **Figure 2**). Consequently, a conversion between dsDNA and ssDNA can be monitored through a change in total DNA length. The number of nucleotides converted can be obtained by using the difference between the lengths of ssDNA and dsDNA. By attaching the DNA to the surface of the flow cell by the 5' tail, leading-strand synthesis can be detected by a shortening of the lagging-strand DNA (**Figure 3**).

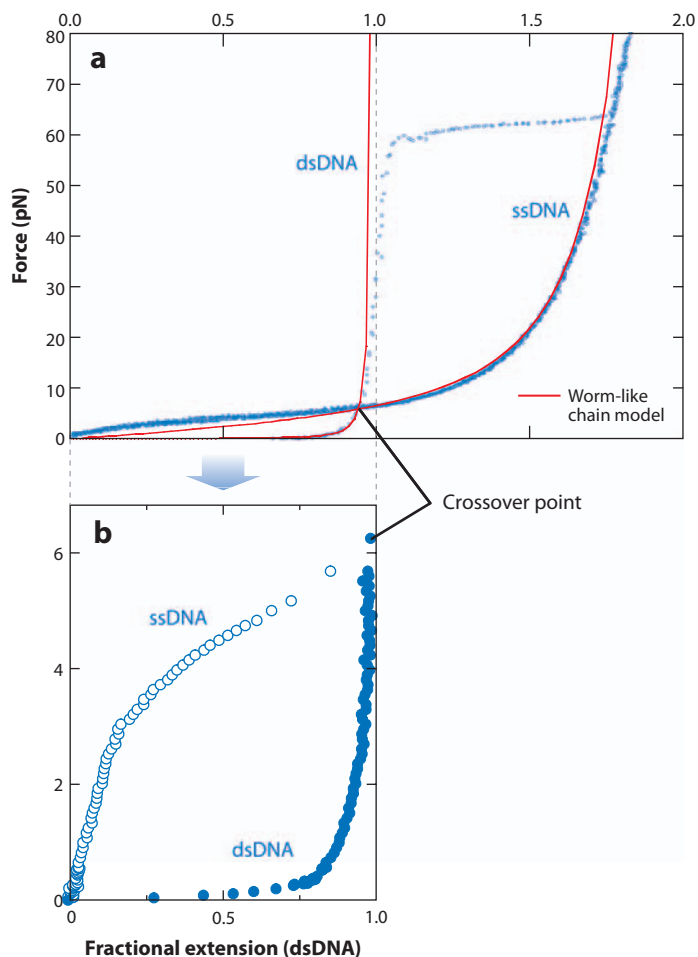


Figure 2

Force-extension data for double-stranded DNA (dsDNA) and single-stranded DNA (ssDNA), visualized at different force regimes [high forces in panel *a* using optical trapping (117); low forces in panel *b* using flow stretching (64)]. Because of the different force-extension behaviors of ssDNA and dsDNA, any conversion between the two forms of DNA can be observed as a change in length. At forces higher than the crossover force (~ 6 pN), dsDNA is shorter than ssDNA; at forces lower than ~ 6 pN, ssDNA is shorter than dsDNA. The red curves in panel *a* correspond to fits to the experimental data using the worm-like chain model. (Reproduced with permission from Reference 112).

A single gp4-DNA polymerase was assembled on the fork in the presence of dNTPs, but in the absence of Mg^{2+} , which is required for both DNA polymerase and helicase activity. Subsequent washing of the flow cell for several minutes ensured that proteins that could exchange or compete with the proteins at the

fork are not present in solution. Leading-strand synthesis reactions were initiated by the addition of Mg^{2+} and dNTPs. The observed rate of leading-strand synthesis (164 bp s^{-1}) (64) is comparable to the synthesis rate by an individual DNA polymerase ($100\text{--}200 \text{ nt s}^{-1}$) (117), but faster than the unwinding rate by a single helicase (30 bp s^{-1}) (48).

The processivity is dramatically increased compared with that of the individual enzymes [17,000 for the gp4-DNA polymerase (64) versus 343 bp for the helicase (48) and 500–1000 nt for the DNA polymerase (64, 117)], underscoring the stability of the helicase-DNA polymerase complex on forked and primed DNA. The existence of a strong physical interaction between the two complexes was validated by monitoring leading-strand synthesis catalyzed by the polymerase associated with a gp4 truncated at its C terminus by 17 residues. The negatively charged C-terminal tail of gp4 is thought to be at least partially responsible for the interaction with the DNA polymerase (65). The rate of leading-strand synthesis remained the same, whereas the processivity in the absence of an interaction with gp4 was reduced to 5 kb (38).

Similar experiments were performed on the leading-strand synthesis complex of *E. coli* (104). Here also, the processivity of the leading-strand synthesis complex (10.5 kb) was significantly higher than that of the DNA polymerase alone (1.4 kb), pointing to an increase in stability when more factors that provide additional protein-protein and DNA-protein interactions are added. The processivity of the *E. coli* leading-strand synthesis complex as measured in the single-molecule experiments was significantly lower than those measured in solution-phase experiments (77) (10 kb versus >50 kb, respectively). The length of final DNA products as observed in solution-phase experiments might contain the contributions of multiple synthesis events on an individual DNA molecule, whereas single-molecule experiments allow for an unequivocal discrimination between one processive event and multiple successive ones.

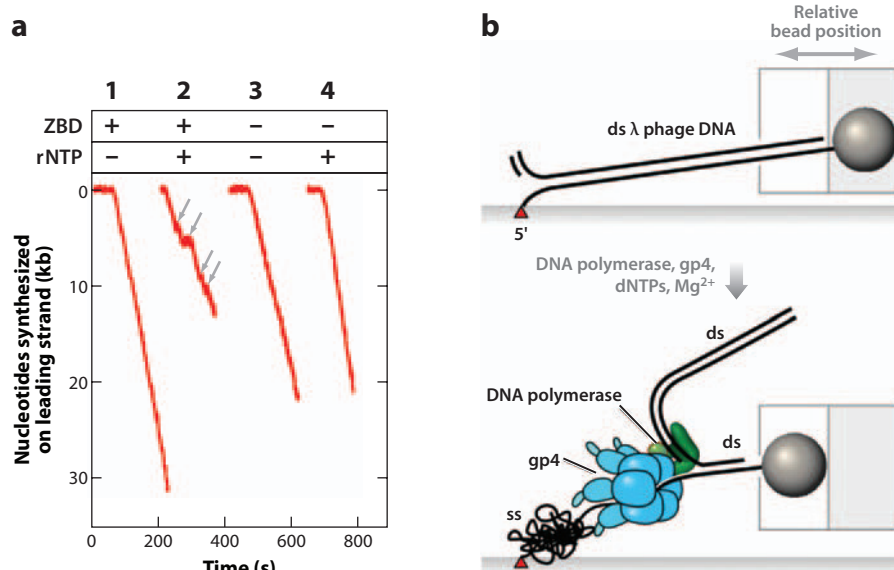


Figure 3

Single-molecule observation of leading-strand synthesis by T7 replication proteins. (a) Single-molecule trajectories of DNA length show shortening corresponding to the leading-strand synthesis and the concomitant conversion of double-stranded DNA (dsDNA) to single-stranded (ssDNA) at the lagging strand. Presence of ribonucleotides and the gp4 zinc-binding domain (ZBD) required for primase activity results in pauses (gray arrows) in the trajectories, suggesting that primer synthesis transiently halts leading-strand synthesis. (b) Schematic depiction of the events in panel a. After introduction of replication proteins and nucleotides, the T7 gp4 helicase unwinds DNA and the T7 DNA polymerase synthesizes on the leading strand. The lagging strand is anchored to the surface and remains in single-stranded form, resulting in a shorter DNA tether. (Reproduced with permission from Reference 64).

DNA Primase Activity

A hallmark of coordinated replication is the tight coupling of the continuous leading-strand synthesis with the discontinuous production of Okazaki fragments at the lagging strand (15, 55, 62, 63). For the system to be coupled, the overall rate of DNA synthesis on the leading strand needs to equal that on the lagging strand. The RNA polymerization rate of the primase is orders of magnitude lower (29, 95, 102) than the rate of synthesis by the DNA polymerases at the fork. Furthermore, the recycling of the lagging-strand DNA polymerase from a finished Okazaki fragment to a new primer site is a process that takes up to a few seconds, enough time for the leading-strand DNA polymerase to synthesize several hundred nucleotides (101). It is not well understood how these slow enzymatic steps can take

place at the lagging strand without losing coordination with the continuous and fast leading-strand synthesis (62, 93, 108). Does the lagging-strand DNA polymerase synthesize faster than the leading-strand DNA polymerase to compensate for the time lost during polymerase cycling (14, 82)? Or does leading-strand synthesis transiently halt to prevent it from outpacing lagging-strand synthesis (64)?

These questions were addressed by monitoring leading-strand synthesis in the presence of an active primase synthesizing ribonucleotide primers on the lagging strand using the single-molecule flow-stretching assay described above (64). A peculiarity of the T7 replication system is that the primase domain is located on the N-terminal half of the gp4 and thus is physically coupled to the helicase domain (28, 34). The primase domain itself consists of two

domains, connected by flexible protein linkers (53, 90). The 7-kDa N terminus, the zinc-binding domain, mediates recognition of the primer sequences on the template DNA and delivery of the newly synthesized primer into the active site of the DNA polymerase (1, 52). The RNA polymerase domain contains the active site where the RNA primer is synthesized. T7 gp4 unwinds DNA while encircling the lagging-strand DNA, thus allowing its primase domain to lay down primers on the lagging strand that can be used to prime the second, lagging-strand DNA polymerase (**Figure 1a**). The position of the primase active site on the outside of the helicase (114) allows the helicase to continue unwinding while a primer is synthesized on the lagging strand.

Enabling primase activity by adding ribonucleotides to the leading-strand synthesis reaction mixture resulted in the appearance of short pauses in the single-molecule leading-strand synthesis traces (**Figure 3**) (64). The absence of pausing when omitting ribonucleotides or using an N-terminally truncated gp4 confirmed that the pausing observed in the reactions with the full-length gp4 and ribonucleotides is dependent on primase activity. Also, a comparison of multiple leading-strand synthesis traces that display primase activity showed that the pauses tended to occur at reproducible positions on the lambda phage DNA that were consistent with the specific sequence requirement of the T7 primase. The precise molecular mechanism underlying the halting of leading-strand synthesis by the primase activity is still unclear.

Even in systems where the primase is not covalently linked to the helicase, the intimate interaction between the primase and helicase is of crucial importance for regulating the coordination between leading- and lagging-strand synthesis (15, 18, 116). In the *E. coli* replication system, the DnaG primase needs to transiently associate with the DnaB helicase to synthesize a primer and deposit it on the lagging strand (18). The frequency of interaction between DnaG and DnaB controls the length of the Okazaki fragments (116, 124). Single-molecule experiments on the *E. coli* leading-strand

synthesis complex demonstrated that the association between DnaG and DnaB resulted in a cessation of fork progression (104), analogous to the pausing behavior observed in the T7 system (64). The reduction in processivity was cooperative with respect to the primase concentration when primer synthesis was allowed (104). This observation supports previously proposed interactions between the zinc-binding and RNA polymerase domains of adjacent DnaB-bound DnaG monomers (18, 19). Crystal structures of *Bacillus stearothermophilus* DnaB and helicase-binding domain of DnaG show three DnaG monomers interacting with a DnaB hexamer, supporting the hypothesis that the primase monomers interact in *trans* (3). These and similar interactions between primase monomers in the T7 replication system have been proposed to serve as a mechanism of regulating primase activity (18, 19, 66, 126).

Recent single-molecule experiments have demonstrated the existence of an alternative mechanism to couple primase activity to leading-strand synthesis (72, 82). The formation of a small, ssDNA loop between helicase and primase would allow helicase-mediated unwinding to continue while the primase remains bound to the lagging strand to synthesize a primer. As a result, formation and growth of such a priming loop allow the leading-strand complex to continue without pausing and having to wait for the relatively slow primase activity. Single-molecule FRET studies using short, forked DNA substrates demonstrated the existence of priming loops in the T7 leading-strand synthesis reaction (82). The authors also presented evidence that the lagging-strand DNA polymerase synthesizes DNA faster than the leading-strand polymerase, a necessary requirement for the lagging-strand polymerase to make up for the time lost during primer synthesis. Magnetic tweezers were used to study priming kinetics in the T4 replication system (72). The authors visualized the unwinding of a hairpin DNA substrate and demonstrated that short, ssDNA loops were formed upon interaction of the T4 primase with the helicase

and subsequent primer synthesis. No pauses were observed in either study, underscoring the need for more work to understand the roles of primase-induced pausing and looping in coupling leading-strand synthesis to primer production.

Lagging-Strand Synthesis and Replication-Loop Formation

The activity of fully assembled replisomes, supporting coupled leading- and lagging-strand synthesis, was visualized by coupling the 5' tail of a rolling-circle substrate to the surface of a glass microscope coverslip and

imaging the growing, flow-stretched dsDNA product of lagging-strand synthesis through use of a DNA intercalating dye (**Figure 4**) (105). The ease of this experimental design and its ability to visualize many hundreds of individual replication reactions simultaneously allowed for a thorough characterization of the distribution of fork rates and processivities for the *E. coli* and T7 replication systems. The O'Donnell group (122) applied this technique to the study of elongation kinetics of preassembled *E. coli* replisomes and demonstrated that lagging-strand synthesis slows down overall fork progression. This observation is consistent with a model in which primase activity on the

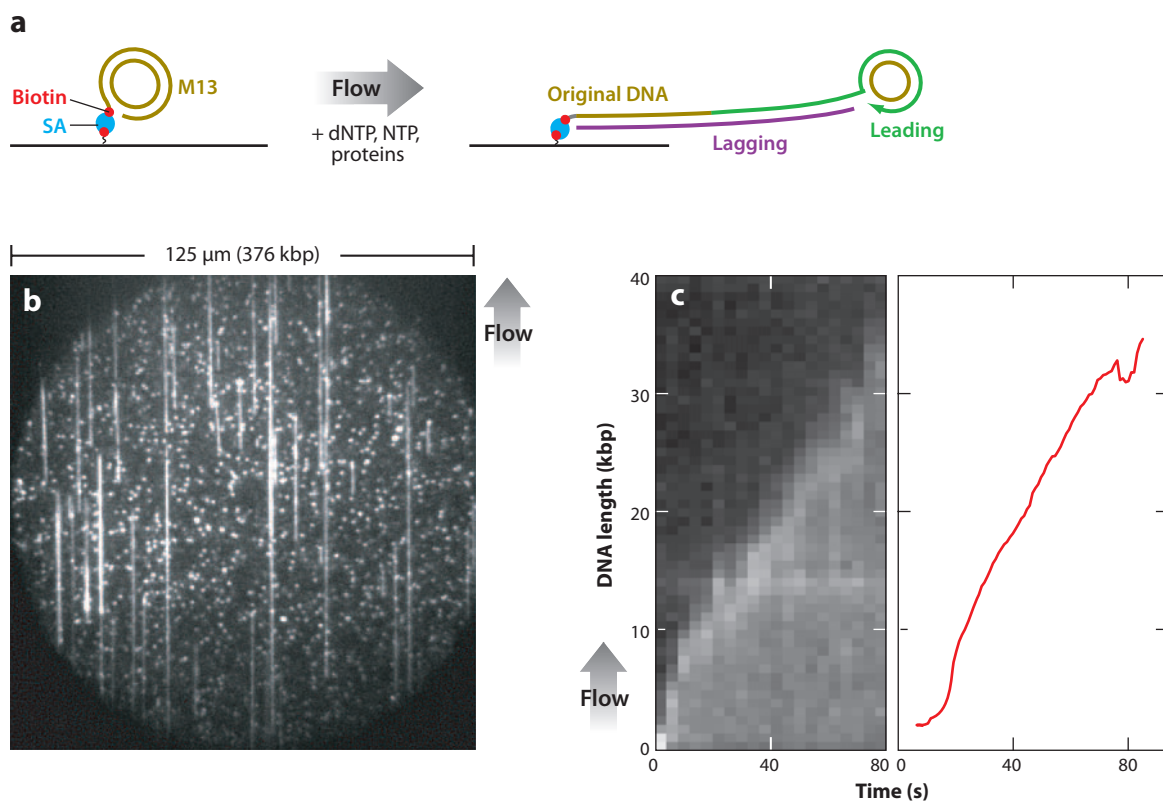


Figure 4

Single-molecule rolling-circle fluorescence imaging assay to monitor coupled replication. (a) Schematic depiction of assay. An M13 rolling-circle template is tethered to the surface of a microscope coverslip, replication proteins are introduced, and the flow-stretched products are imaged in real time by using an intercalating stain at low concentrations. (b) Snapshot of replication intermediates. Flow is from bottom to top. (c) Kymograph of an individual M13 that is replicated by the *Escherichia coli* replisome (left). Tracking the position of the free end of the DNA tether results in a trajectory from which rate and processivity parameters can easily be obtained (right). (Reproduced with permission from Reference 105). Abbreviations: dNTP, deoxyribonucleoside triphosphate; SA, streptavidin.

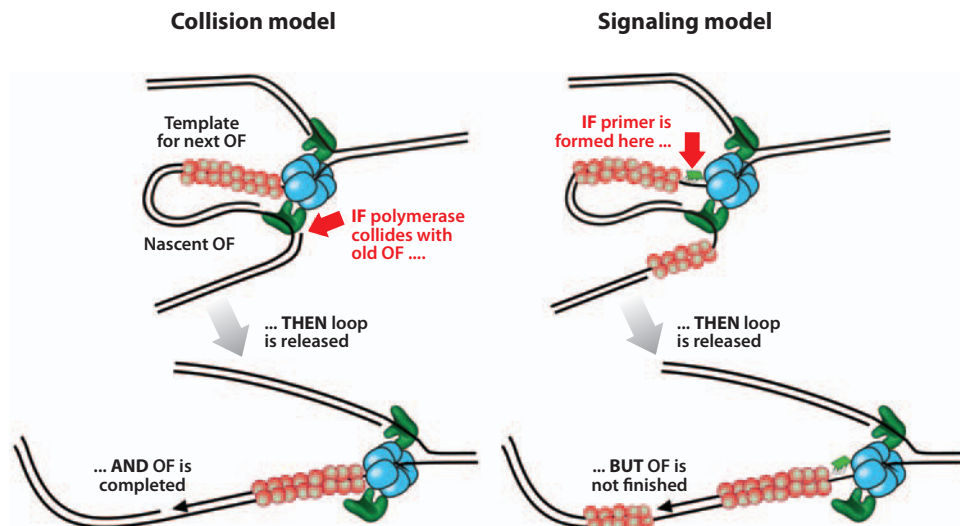


Figure 5

Mechanisms of replication loop release. (*Left*) In the collision model, the loop is released when the lagging-strand DNA polymerases completes the nascent Okazaki fragment (OF) and collides with the 5' end of the previous OF. (*Right*) In the signaling model, primer synthesis signals loop release.

lagging strand slows down or transiently halts leading-strand synthesis.

One important property that gives the replisome its efficiency is its ability to support many cycles of priming and Okazaki fragment synthesis. It is not well understood what triggers the dissociation of the polymerase from the 3' end of a nascent Okazaki fragment to allow this cycle to restart. Two models can be envisioned (**Figure 5**): (*a*) a collision-directed trigger, in which the DNA polymerase dissociates from the 3' end of the Okazaki fragment immediately after encountering the 5' terminus of the previous fragment (2, 36, 37, 61, 67, 123); and (*b*) a primase-directed trigger, in which the DNA polymerase dissociates when a primer is synthesized (116, 121). In the latter model, primer synthesis initiates every lagging-strand cycle, and its frequency determines the length distribution of the Okazaki fragments (124).

Recent single-molecule studies enabled the visualization of the formation of replication loops on the lagging strand by monitoring transient shortenings in flow-stretched DNA undergoing coupled replication (**Figure 6**) (39).

The length measurements of individual DNA substrates reveal that the replication loop release and the initiation of a new one are separated by a lag phase, a step unobservable in bulk-phase assays, in which the overall length of DNA does not change. The appearance of a lag time after replication loop release suggests the requirement of intermediary steps prior to the formation of a new replication loop. From these single-molecule experiments, it appeared that half the time the replisome was not engaged in the production of a replication loop. This observation is consistent with electron microscopy studies on intact replisomes, which revealed that only half of the active replisomes contained a loop (17, 62, 79, 86). Analysis of the distributions of loop sizes and lag times between loops reveals that initiation of primer synthesis and the completion of an Okazaki fragment each serves as a trigger for loop release. The presence of two triggers may represent a fail-safe mechanism that ensures the timely reset of the replisome after the synthesis of every Okazaki fragment (39).

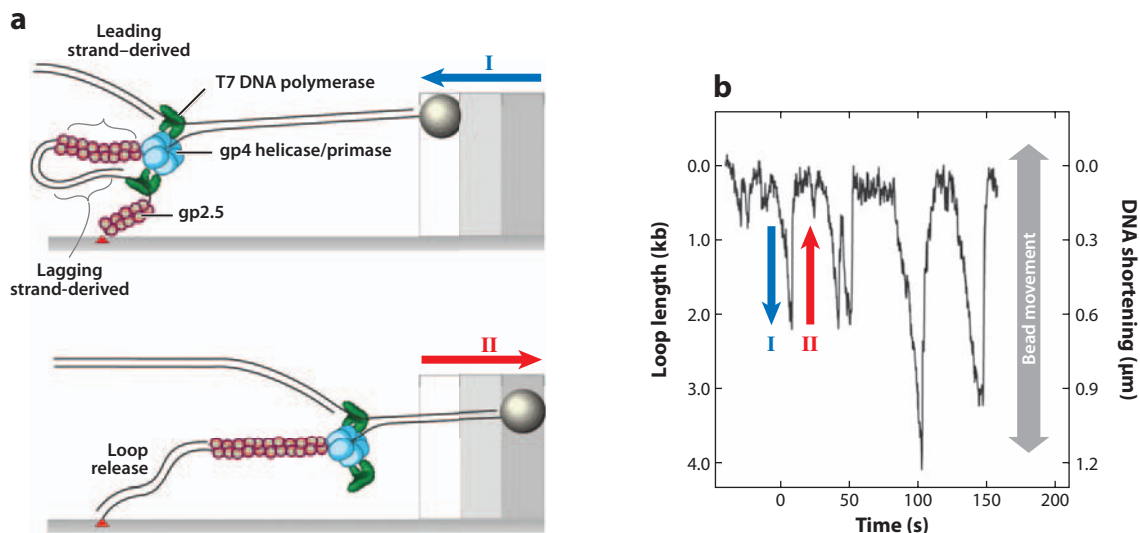


Figure 6

Single-molecule observation of replication loop formation by the T7 replisome. (a) Schematic depiction of experimental design. The presence of the T7 gp2.5 single-stranded DNA (ssDNA) binding protein abrogates the length difference between ssDNA and double-stranded DNA (dsDNA). The production and release of loops in the lagging strand cause the measured length of the DNA to shorten gradually and lengthen abruptly, respectively. (b) Single-molecule trajectory showing repeated loop formation and release events. The loop lengths and the duration of the lag phases between loop events are used to unravel the different timing mechanisms of loop release (see text). (Reproduced with permission from Reference 39.)

FUTURE DIRECTIONS

The last few years have seen a rapid development of single-molecule biophysical tools and their application to the study of the replisome. The mechanical stretching of individual DNA molecules (12, 13, 76, 112) has allowed DNA unwinding and synthesis to be visualized and has finally resulted in tools for studying intact replisomes. The development of single-molecule fluorescence techniques, in particular FRET, has allowed one to dissect protein-protein and DNA-protein interactions in intimate kinetic detail (10, 35, 50, 73, 127). The next important step will be to combine the mechanical manipulation of individual DNA molecules with the use of single-molecule fluorescence imaging (43, 111). Measuring unwinding and synthesis by tracking the length of a DNA molecule while imaging the fluorescence of labeled replication proteins at the fork will allow researchers to relate function (the

enzymatic activities at the fork) to structure (the architecture of the replication fork).

Ultimately, these techniques will enable us to address a number of broader issues inaccessible by ensemble-averaging techniques: What is the molecular mechanism of the coupling between primase activity and leading-strand synthesis? What determines the rate of primer synthesis and thus the length of Okazaki fragments? How does the replisome deal with barriers on the DNA, such as transcription complexes? What is the order of events that lead to replication fork restart after encountering a lesion or obstacle? If we can answer these questions for prokaryotic model systems, then we will provide a mechanistic framework that allows us to begin thinking about the function and regulation of the eukaryotic replisome, a complex with similar function but significantly higher complexity.

Where the dynamic functioning of the prokaryotic replisome is unclear, the

composition and organization of the eukaryotic fork are even larger mysteries (47). The biochemical complexity of the eukaryotic fork stands in the way of an *in vitro* reconstitution of the replication reaction and makes mechanistic studies difficult to perform. Instead, individual eukaryotic replication forks could be studied in cell extracts (24, 110), an environment that

closely mimics physiological conditions but still allows the use of mechanical manipulation tools and single-molecule fluorescence imaging. Combined with complementary biochemical, genetic, and structural approaches, these methods will bring us closer to a full understanding of the different components of the replisome and their dynamic properties.

SUMMARY POINTS

1. A large number of single-molecule techniques have become available to study the dynamic behavior of nucleic acid enzymes: force techniques such as magnetic tweezing, optical trapping, and flow stretching, and fluorescence techniques such as single-molecule FRET and colocalization imaging.
2. Single-molecule tools are now routinely applied to the study of individual components of the replication machinery and have significantly contributed to our understanding of the functioning of these proteins. In particular, these techniques have elucidated the mechanisms of helicase-mediated unwinding, polymerase-mediated proofreading, and binding modes of SSBs to ssDNA.
3. The ability of single-molecule tools to visualize transient intermediate states makes them ideally suited to study the complex organization and dynamics of multiprotein replication machinery. Single-molecule studies of the replisome have already resulted in new insights into replisome assembly and the coupling between leading- and lagging-strand synthesis.
4. The complexity of the replisome and the rarity of some of its salient intermediate states argue for the use of multiplexed methods, in which many individual replication reactions can be observed simultaneously.

FUTURE ISSUES

1. To relate enzymatic activity of the replisome with its structural organization and dynamics, there is a need for techniques that combine the ability to mechanically manipulate DNA substrate and observe fluorescence of labeled proteins at the fork.
2. Techniques that allow fluorescence detection of individual proteins while observing enzymatic activity by DNA-length measurements will enable us to correlate structural with functional properties of the replisome. Such methods are needed to inform on the composition of the replisome during replication: How many polymerases are associated with the complex? Are polymerases and processivity factors recycled after completion of Okazaki fragments?
3. The further development of chemical and genetic labeling strategies is needed to fluorescently tag the replication proteins at any point of interest. Such a flexibility in labeling the replisome will enable the observation of FRET between different components at the fork and thus will directly visualize intra- or intermolecular dynamics during fork progression.

4. To understand the functioning of the significantly more complex eukaryotic replication machinery, methodology that allows the manipulation and study of individual replisomes inside the living cell or in cellular extracts needs to be developed.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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